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**(54) Title:** DNA FRAGMENTS AND DNA PROBES AND PRIMERS BASED THEREON**(57) Abstract**

This invention relates to a DNA fragment derived from a methicillin-resistant *Staphylococcus* strain as well as to a DNA probe, based on such a DNA fragment, for the detection of *Staphylococcus* bacteria that are resistant to methicillin and related penicillins insensitive to  $\beta$ -lactamase and to a DNA-primer, based on such a DNA fragment, for DNA amplification by means of a polymerase chain reaction. The DNA fragment according to the invention consists essentially of or is complementary to the sequence shown in the sequence list under SEQ ID NO:1 or a related sequence, such as one of the sequences shown in the sequence list under SEQ ID NO's:2-8, or part of such a sequence having a length of at least 14 nucleotides. The invention also comprises diagnostic kits and processes on the basis of such DNA fragments.

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Title: DNA fragments and DNA probes and primers based thereon

The present invention relates to a DNA fragment derived from a methicillin-resistant Staphylococcus strain as well as to a DNA probe based on such a DNA fragment and adapted for the detection of Staphylococcus bacteria that are resistant to methicillin and related penicillins insensitive to  $\beta$ -lactamase and to a DNA-primer based on such a DNA fragment and adapted for DNA amplification by means of a polymerase chain reaction.

Of the Staphylococcus bacteria Staphylococcus aureus is the most important bacterium pathogenic to man. The appearance of methicillin-resistant strains of this bacterium is a serious problem in antibacterial chemotherapy (1,2). Such strains are resistant to methicillin and also to closely related penicillins insensitive to  $\beta$ -lactamase, such as cloxacillin, flucloxacillin, etc. In the following description these strains will be designated, for brevity's sake, as methicillin-resistant strains.

It has been found that methicillin resistance is connected with the presence of a unique penicillin-binding protein (PBP2a or PBP2'), which is apparently not present in sensitive Staphylococcus strains. The genetic determinant for methicillin resistance (mec) is located in the chromosomal DNA (3). Various publications are devoted to the involvement of mec and possibly a second factor in the methicillin resistance of Staphylococcus strains (4-10). Experiments in which the mec gene of a methicillin-resistant S. aureus isolate was inactivated by insertion of a transposon have shown that the mec gene is necessary for resistance (7). Methicillin-resistant strains of Staphylococcus epidermidis, S. haemolyticus, S. hominis, S. simulans and S. saprophyticus contain a penicillin-binding protein comparable to the product of the mec gene (8,9) and responsible for resistance to methicillin. Involved in the regulation of the expression of methicillin resistance is a second element designated by mecR (10), which, however, does not occur in all the resistant strains.

The method conventional in practice for demonstrating methicillin-resistant Staphylococcus strains consists in culturing the strain on agar plates in the presence of methicillin for about 48 hours.

5 In addition to this conventional method, so-called DNA probes are increasingly used in clinical laboratories. DNA probes are small segments of single-stranded nucleic acid labelled with, e.g., an enzyme or a radioisotope which very specifically bind to complementary nucleic acid sequences  
10 (hybridization). The detection of bound label indicates the presence of the pathogen to be demonstrated in a clinical sample. Thus, probes have been developed for detecting, e.g., bacteria, viruses, protozoa, and even fungi. A number of probe kits are already commercially available. A problem in the  
15 development of a probe is that it must be sensitive and very specific. The aim is to develop a smallest possible probe being sufficiently specific.

A probe for the detection of resistance to a certain antibiotic is known from, e.g., French patent application  
20 2,602,794. This application relates to a DNA fragment containing at least part of the tetM gene coding for a tetracycline-resistance protein and the nucleotide sequence of which is given. Such a fragment can be used for the preparation of a DNA probe for the detection of tetracycline  
25 resistance in a cell culture. The only probe found useful is a rather large DNA fragment of 850 bp.

Also for distinguishing methicillin-resistant and methicillin-sensitive Staphylococcus strains, hybridization analysis by means of DNA probes has been proposed (11). The  
30 DNA probe used therein is a 3.9 kb HindIII restriction fragment of chromosomal DNA of a methicillin-resistant Staphylococcus aureus strain, which fragment hybridized with the 3.5 kb BglII restriction fragment previously described by Beck et al (5). The 3.9 kb DNA probe hybridized with  
35 chromosomal DNA of all the selected methicillin-resistant Staphylococcus haemolyticus and Staphylococcus epidermidis

strains, whereas no hybridization occurred with the negative hybridization controls, namely a methicillin-sensitive Staphylococcus haemolyticus isolate and a methicillin-sensitive Staphylococcus epidermidis isolate.

5        However, the great length (3.9 kb) and unknown base sequence of this probe impede development of sensitive and specific tests. The same applies to the recently proposed (12) probe based on a 1.1 kb BglII-XbaI fragment of the mec gene coding for PBP2a.

10        In clinical practice, there is a great need for a sensitive and specific DNA probe for the detection of methicillin-resistant Staphylococcus bacteria, in particular Staphylococcus aureus, but also Staphylococcus epidermidis, Staphylococcus saprophyticus, Staphylococcus haemolyticus,  
15        Staphylococcus warneri, Staphylococcus hominis, Staphylococcus simulans, etc. Similarly, there is a great need for DNA fragments adapted for use as DNA primers in a PCR technique (Polymerase Chain Reaction) for a specific amplification of DNA of methicillin-resistant Staphylococcus bacteria.

20        The invention satisfies these needs by means of a DNA fragment derived from a methicillin-resistant Staphylococcus strain and consisting essentially of or being complementary to the nucleotide sequence shown in the sequence list under SEQ ID NO:1 or a related nucleotide sequence, such as one of the  
25        nucleotide sequences shown in the sequence list under SEQ ID NO's:2-8, or part of such a nucleotide sequence having a length of at least 14 nucleotides.

      The DNA fragments according to the invention are derived from a methicillin-resistant Staphylococcus strain. In  
30        principle, the invention is not limited to DNA fragments derived from a certain species of Staphylococcus bacteria. Thus, the DNA fragments may be derived from a methicillin-resistant strain of Staphylococcus aureus, Staphylococcus epidermidis, Staphylococcus saprophyticus, Staphylococcus  
35        haemolyticus, Staphylococcus warneri, Staphylococcus hominis, Staphylococcus simulans, etc. The DNA fragments with the

nucleotide sequences according to SEQ ID NO's:1-8 are all derived from methicillin-resistant Staphylococcus aureus strains. Yet they can also be used for the detection of methicillin-resistant strains of other species of

5 Staphylococcus bacteria, which is due to the great homology between the relative sequences of the different Staphylococcus species.

The nucleotide sequence shown in the sequence list under SEQ ID NO:1 is derived from the methicillin-resistant S.

10 aureus strain 05723. For the purpose of the invention a nucleotide sequence related thereto is the corresponding sequence in other methicillin-resistant Staphylococcus strains, both methicillin-resistant strains of the species S. aureus and methicillin-resistant strains of other

15 Staphylococcus species (such as the above-mentioned species). Examples of such related sequences are given in the sequence list under SEQ ID NO's:2-8, which are derived from the methicillin-resistant S. aureus strains 00646, A216, 02599, 214, 215C, 06231 and 1335, respectively. As shown by these

20 examples, there is a large degree of homology between these sequences, but other significant differences in length may also occur: the DNA sequences derived from the S. aureus strains 02599, 214, 215C, 06231 and 1335 prove to be about 68 nucleotides shorter than those of the other examples shown.

25 The DNA sequences found, which (like the sequences complementary thereto) may all function as a suitable probe for the detection of methicillin resistance in Staphylococcus aureus, have been compared with all the sequences occurring in the EMBL (European Molecular Biology Laboratory) data bank.

30 Homology has been found with three sequences:

1. pp5mer (13), a partial plasmid DNA sequence involved in mercury resistance of Staphylococcus aureus;
2. pp5cada (14), a partial plasmid DNA sequence involved in cadmium resistance of Staphylococcus aureus;
- 35 3. Tn4003 (15), a transposon found in Staphylococcus aureus and giving rise to trimethoprim resistance.

The sequences shown in the sequence list under SEQ ID NO's:1-8 can be subdivided into two groups, the first consisting of DNA fragments having a length of about 333 bp and the second consisting of DNA fragments lacking a sequence of about 68 bp (nucleotides 155-222). In the fragments of about 333 bp three parts can be distinguished: nucleotides 1-154 (Part I), nucleotides 155-222 (Part II. which part is absent in the DNA fragments of the second group) and nucleotides 223-333 (Part III). The observed homology with pp5mer extends over all the three parts; that with pp5cada and Tn4003 only over Part III. Part III proves to form part of insertion sequence IS257, also designated by IS431, occurring in transposon Tn4003.

The invention provides DNA probes and primers based on DNA fragments as defined above. Generally, DNA probes designate labelled DNA fragments adapted for the detection of complementary sequences, but in a broader sense they can also designate non-labelled DNA fragments capable of specifically binding to complementary sequences. Suitable labelling methods are known to those skilled in the art. By way of example, mention is made of labelling with radioisotopes, enzymes, fluorescent substances, pigments, the biotin-avidin system, etc. Both labelled and unlabelled DNA probes according to the invention can be used for the detection of Staphylococcus bacteria resistant to methicillin and related penicillins insensitive to  $\beta$ -lactamase by means of hybridization analysis.

A classical hybridization analysis makes use of a labelled probe which, if necessary after a denaturation treatment, is contacted with the denatured target DNA bound to a solid carrier (such as nitrocellulose). The hybridization is followed by detection of the label, e.g., of a radioisotope by autoradiography by means of an X-ray film, of an enzyme label by means of a substrate of the enzyme, etc.

An alternative hybridization analysis uses both a labelled and an unlabelled probe which are complementary to different parts of the target DNA, the latter probe serving to bind the

target DNA to a solid carrier and the former probe serving to provide the bound target DNA with a detectable label. SEQ ID NO's:15 and 16 are examples of sequences that could function as the unlabelled "collecting" probe and the labelled  
5 "marking" probe, or conversely. Polymer particles (e.g., polystyrene globules) or magnetic particles could function as a solid carrier, in which connection different methods can be used to bind the unlabelled probe to the carrier (e.g., by means of the avidin-biotin system, etc.). The different steps  
10 (including a prehybridization with hybridization buffer to inhibit aspecific bindings and washing treatments after the hybridization step to remove unbound probe) and the employed reagents and reaction conditions are fully known to those skilled in the art and need no further explanation.

15 The invention therefore also provides a diagnostic test kit for detection in a sample of Staphylococcus bacteria that are resistant to methicillin and related penicillins insensitive to  $\beta$ -lactamase by means of hybridization analysis, comprising at least a (labelled) DNA probe according to the  
20 invention as well as one or more further means required for hybridization analysis, such as a denaturation liquid, a hybridization liquid, a washing liquid, a solid carrier, a hybridization vessel and label detecting means.

The invention also provides a process for the detection in  
25 a sample of Staphylococcus bacteria that are resistant to methicillin and related penicillins insensitive to  $\beta$ -lactamase by means of hybridization analysis, which comprises isolating the DNA from the bacteria in the sample, contacting the isolated DNA under hybridization conditions with at least a  
30 (labelled) DNA probe according to the invention, washing non-hybridized probe and analyzing any hybridization by label detection.

The DNA probe according to the invention is preferably based on Parts I, II and III together, or on Parts I and III  
35 together. Part III alone forms a very suitable probe. Less suitable are probes on the basis of Parts I and II alone or

Parts I and II together. Examples of relatively short probes are SEQ ID NO's:15 and 16.

DNA primers are DNA fragments that are complementary to part of a "target" DNA and, after hybridization thereto, may  
5 serve as starting-point for DNA polymerase which, in the presence of the necessary building materials, synthesizes the chain complementary to the target DNA. By selecting two suitable primers in a DNA fragment according to the invention, the presence in a sample of DNA of methicillin-resistant  
10 Staphylococcus strains can be determined by means of the PCR method in a very sensitive and specific manner. The different steps, reagents and reaction conditions are known to those skilled in the art and need no detailed explanation. This also applies to the methods by which the result of the PCR can be  
15 analyzed, e.g., separation according to length of the resulting DNA fragments and visualization thereof by means of ethidium bromide, or hybridization analysis of the resulting DNA fragments.

In general, primers will be relatively short, provided  
20 they are still sufficiently specific for the contemplated target DNA. DNA fragments having a length of 14 nucleotides are often sufficient, but slightly longer fragments, say from 15 to 40, preferably from 18 to 32 nucleotides, are preferred. Such relative short DNA fragments may also be used for DNA  
25 probes, as elucidated by means of SEQ ID NO's:15 and 16 (but also larger fragments, particularly the complete sequences SEQ ID NO's:1-8 as given in the sequence list, are suitable as probes).

Examples of suitable primer sets are the DNA fragments  
30 shown in the sequence list under SEQ ID NO's:9 and 10, or SEQ ID NO's:11 and 12. Within a primer set one of the primers may be based on a sequence in Part I of the DNA fragments according to the invention, whereas the other primer is based on a sequence occurring in Part III. It is preferred, however,  
35 that both primers of a primer set are based on a sequence occurring in Part III. Very suitable is a primer pair, of

which the first primer is SEQ ID NO:13 and the second primer is SEQ ID NO:14.

The invention therefore also provides a diagnostic test kit for the detection in a sample of Staphylococcus bacteria that are resistant to methicillin and related penicillins insensitive to  $\beta$ -lactamase by means of PCR analysis, comprising a DNA primer set according to the invention, as well as one or more further means required for PCR analysis, such as a polymerase, a polymerization liquid, an oil overlay, a reaction vessel and means for detecting the amplified DNA.

The invention also provides a process for the detection in a sample of Staphylococcus bacteria that are resistant to methicillin and related penicillins insensitive to  $\beta$ -lactamase by means of PCR analysis, which comprises isolating, if required, the DNA from the bacteria in the sample, subjecting the DNA that may or may not have been isolated to several PCR cycles using a DNA primer set according to the invention, and analyzing the amplified DNA.

In the case of detection of methicillin-resistant Staphylococcus aureus strains it is preferred to carry out a combined PCR analysis with both a set of primers according to the invention and a set of primers which is specific for protein A, a protein specific for Staphylococcus aureus. By simultaneously using primers according to the invention and protein A-specific primers, an internal control of the PCR will be possible. If the analysis of the amplified DNA indicates that no PCR product has been formed with the primers according to the invention, the formation of PCR product with the protein A-specific primers may serve to prove that the PCR has proceeded well, so that the negative result may be attributed to the absence of methicillin-resistant strains. A suitable set of protein A-specific primers is shown in the sequence list with SEQ ID NO's:17 and 18. Also the primers SEQ ID NO's:18 and 19 form a suitable primer pair.

The invention further provides a plasmid, consisting essentially of a bacterial cloning vector including an

insertion consisting of a DNA fragment according to the invention, and bacteria containing at least one such plasmid.

A DNA fragment with the nucleotide sequence according to SEQ ID NO:1 is contained in plasmid pAA29-1 in E. coli DH5 $\alpha$ F',  
5 which strain was deposited with Centraal Bureau voor Schimmelcultures (CBS) of Baarn, The Netherlands, on November 15, 1989 under access no. CBS 588.89. A probe based on this DNA fragment is very specific. With all the examined Staphylococcus strains this probe gave a perfect correlation  
10 with methicillin resistance.

A DNA fragment according to the invention may be obtained by the following process:

- a) partial digestion of purified DNA of a methicillin-resistant Staphylococcus strain with, e.g., Sau 3AI,
- 15 b) hybridization of the resulting fragments with labelled DNA fragments of a methicillin-sensitive Staphylococcus strain,
- c) collecting the free DNA fragments,
- d) repetition of steps b) and c),
- e) ligation of the resulting fragments in a plasmid and
- 20 transformation of a suitable bacterial strain with this plasmid,
- f) culturing the transformants and isolation of the DNA from the transformants,
- g) hybridization of the isolated DNA with labelled DNA of a
- 25 small number of methicillin-resistant and methicillin-sensitive Staphylococcus strains,
- h) selection of the transformants only showing hybridization with DNA of the methicillin-resistant strain or strains,
- i) digestion with, e.g., PstI and EcoRI of the DNA of the
- 30 selected transformants,
- j) labelling in a conventional manner of the digested DNA fragments,
- k) hybridization of the labelled DNA fragments with DNA of a large number of methicillin-resistant and methicillin-
- 35 sensitive Staphylococcus strains,

1) selection of labelled DNA fragments only showing hybridization with DNA of methicillin-resistant strains.

All the steps of this process consist in known per se methods and will be explained further, as far as necessary, in the part entitled "Materials and Methods" or in the Example.

The Staphylococcus strains used in steps a, b, d, g and k may in principle be nearly any Staphylococcus strain or isolate, provided they satisfy the relative criterion of methicillin resistance or methicillin sensitivity required in the different steps.

Step b) preferably consists in hybridization in solution at elevated temperature of the resulting fragments with sheared biotinylated DNA fragments of a methicillin-sensitive Staphylococcus strain.

Step e) preferably consists in ligation of the resulting fragments in the unique BamHI restriction site of pUC18 and transformation of E. coli DH5 $\alpha$ F' with this plasmid.

Step f) preferably consists in culturing the transformants, lysis and DNA fixation on filters.

Step g) preferably consists in Southern hybridization of the isolated DNA with labelled DNA of not more than three methicillin-resistant Staphylococcus strains and not more than three methicillin-sensitive Staphylococcus strains.

The following Example I describes more extensively how to obtain the probe according to the invention. In combination with this process, another process will be described which began with digestion with HindIII but did not lead to a desired probe. The specificity of the probe according to the invention appears from the hybridization tests carried out with it, which tests show a perfect correlation with methicillin resistance.

#### Materials and Methods

a) The isolates of methicillin-resistant Staphylococcus aureus used in the preparation of the probe are described in the following Table A. The isolates of methicillin-resistant

Staphylococcus aureus used in the preparation of the probe and in the hybridization tests are described in Table B.

The MIC's for methicillin and gentamicin were determined using the agar dilution method. The MIC's for methicillin were  
5 determined by inoculation of 100  $\mu$ l of a  $10^8$  cfu/ml bacterial suspension on Mueller-Hinton agar followed by incubation at 30°C. Isolates with MIC > 4 were considered resistant. The MIC's for gentamicin were determined by inoculation of 100  $\mu$ l  
10 of a suspension of  $10^6$  cfu/ml on Isosensitest followed by incubation at 37°C. Isolates with MIC > 4 were considered resistant.

Table A

Employed methicillin-resistant Staphylococcus aureus isolates

Indication	Place of isolation	Gentamicin-resistant
05520	Utrecht	+
02790	Alkmaar	+
02222	Ede	+
1335	Delft	not
determined		
Bm	Amsterdam	+
01024	Hilversum	+
05723	Utrecht <sup>1</sup>	+
00646	Utrecht	+
02599	Beverwijk	-
A217	Utrecht	-
Hk	Amsterdam	+
01234	Amsterdam	+
02752	The Hague	+
A215	Utrecht	+
Pt	Utrecht <sup>2</sup>	not
determined		
01924	Eindhoven	+
Sl	Amsterdam	-
1341	Delft	not
determined		
A216	Utrecht <sup>3</sup>	+
00898	Rotterdam	+
01917	The Hague	+
06231	Utrecht <sup>4</sup>	+
214	London	variable
215C	London	variable
262	London	variable

<sup>1</sup> Imported with a patient from Italy<sup>2</sup> Imported with a patient from France<sup>3</sup> Imported with a patient from Portugal<sup>4</sup> Imported with a patient from Yugoslavia.

Table BEmployed methicillin-sensitive Staphylococcus aureus isolates

Indication	Place of isolation	Gentamicin-resistant
8075	Utrecht	-
9838	Utrecht	-
9848	Utrecht	-
Ps-47-8325	London (NCTC)	not determined
88-9990	Groningen	not determined
88-10019	Rotterdam	not determined
88-10021	Rotterdam	not determined
88-10023	Rotterdam	not determined
W-57-7776	Utrecht	not determined
89-91 (type XII)	Groningen	not determined
89-63 (type XIV)	Groningen	not determined
89-77 (type 187)	Groningen	not determined
13.1	Utrecht	not determined
13.2	Utrecht	not determined
13.3	Utrecht	not determined
13.4	Utrecht	not determined
13.5	Utrecht	not determined
13.6	Utrecht	not determined
13.7	Utrecht	not determined
13.8	Utrecht	not determined
13.9	Utrecht	not determined
13.10	Utrecht	not determined
13.11	Utrecht	not determined
13.13	Utrecht	not determined
13.16	Utrecht	not determined
13.17	Utrecht	not determined
13.18	Utrecht	not determined
13.19	Utrecht	not determined
13.20	Utrecht	not determined
13.51	Utrecht	not determined
13.52	Utrecht	not determined
13.53	Utrecht	not determined
13.54	Utrecht	not determined
13.55	Utrecht	not determined
13.58	Utrecht	not determined
13.59	Utrecht	not determined
13.60	Utrecht	not determined
13.61	Utrecht	not determined
13.62	Utrecht	not determined
13.63	Utrecht	not determined
13.64	Utrecht	not determined

b) Staphylococcus aureus DNA was purified with the method as described by Lindberg et al (16), except that after lysis of the bacteria the suspension was extracted with phenol/chloroform as described by Maniatis et al (17). After  
5 the first extraction the mixture was treated with RNase and once more extracted with phenol/chloroform. Plasmid DNA was purified as described by Maniatis et al, followed by centrifugation with CsCl density gradient (17).

c) Digestions with restriction endonucleases were carried  
10 out according to the prescriptions of the manufacturer (GIBCO Laboratories, Paisley, Renfrewshire, Great Britain).

d) The culture, lysis and fixation of transformants on zeta probe filters has been described previously (18).

The Staphylococcus aureus DNA used as probe was labelled  
15 with  $\alpha$ -<sup>32</sup>P-dCTP (specific activity 800 Ci/mmol; Amersham, Great Britain) using a nick-translation kit from GIBCO. Nick translations were carried out as indicated by the manufacturer. The DNA of the plasmid inserts was labelled with digoxigenin from Boehringer (Mannheim, Germany) using  
20 incubation conditions as indicated by the manufacturer. Souther hybridizations were carried out as described by Maniatis et al (17).

#### Example I

25 A method described by Welcher et al (19) was used to enrich DNA fragments specific for methicillin resistance. In this method, purified DNA from the methicillin-resistant isolate 05723 was partially digested with Sau 3AI, resulting in fragments smaller than 4 kb. In order to find DNA fragments  
30 specific for methicillin-resistant strains, hybridization in solution was carried out using the Sau 3AI restriction fragments and sheared biotinylated DNA fragments of the methicillin-sensitive strain Ps-47-8325. 5  $\mu$ g Sau 3AI fragments and 6  $\mu$ g biotinylated DNA were denatured by heating,  
35 after which they were allowed to hybridize in solution at 62°C. After 4 hours at 62°C a suspension of avidin-agarose

granules was added. The biotinylated probe and the hybrids were centrifuged after 5 min. at room temperature to form a pellet. The free DNA fragments in solution were collected and the above procedure was repeated. The resulting fragments were  
5 ligated in the unique BamHI restriction site of pUC18.

Besides, as an alternative procedure, purified DNA from the methicillin-resistant isolate 05723 and the methicillin-sensitive strain Ps-47-8325 was digested with the restriction endonuclease HindIII and analyzed for agarose gel. The  
10 methicillin-resistant isolate showed extra bands at about 9 kb and 3.5 kb. These fragments were isolated and cloned in the unique HindIII restriction site of pUC18.

Transformation of Escherichia coli DH5 $\alpha$ F' with the above plasmids led to 770 clones containing an insert in either the  
15 BamHI or the HindIII restriction site. The transformants were cultured on zeta-probe filters and selected after lysis and DNA fixation with Southern hybridizations using <sup>32</sup>p-labelled chromosomal DNA from one methicillin-resistant isolate (A216) and three methicillin-sensitive isolates (isolates 8075, 9838  
20 and 9848) as probes. Nine transformants were selected showing hybridization with probe DNA of the methicillin-resistant isolates, but not with probe DNA of the sensitive isolates. The transformants were characterized by digestions with restriction endonucleases. The transformants with an insert in  
25 the HindIII site contain Staphylococcus aureus DNA sequences of about 3.5 kb. The inserts in the BamHI site varied from about 0.1 to 1.5 kb.

The inserts of these transformants were isolated by digestion with PstI and EcoRI and then labelled with  
30 digoxigenin and used in Souther hybridizations for about 24 hours with DNA isolated from 25 methicillin-resistant strains and 41 methicillin-sensitive isolates. The isolates used were clinical isolates from different places (see Materials and Methods). The results of the hybridization tests showed a  
35 consistent hybridization of DNA from one specific clone with DNA from methicillin-resistant isolates. This clone was

obtained from the process in which digesting was effected with Sau 3AI in the first step. The relative DNA insert was about 300 bp long. However, no hybridization of the DNA from this clone occurred with DNA obtained from the 41 methicillin-sensitive isolates. The hybridizations with the inserts of the other clones showed no correlation with the absence or presence of methicillin resistance. A correlation with gentamicin resistance could not be demonstrated of any DNA insert.

10 It appears from the above results that the sequence of about 300 bp as found shows an excellent correlation with the occurrence of methicillin resistance in Staphylococcus strains. This sequence may therefore be used as probe for the detection of methicillin-resistant Staphylococcus strains.

15 The base sequence of this probe derived from S. aureus strain 05723 is shown in the separate sequence list under sequence identification number 1.

For different other methicillin-resistant S. aureus strains the DNA sequence of the corresponding range has been determined. In the sequence list the DNA sequences of the probe range of the S. aureus strains 00646, A216, 02599, 214, 215C, 06231 and 1335 are shown under sequence identification numbers 2, 3, 4, 5, 6, 7 and 8, respectively.

## 25 Example II

The use of the PCR method

A PCR was carried out in the following reaction medium: 10 mM Tris HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.001% (w/s) gelatin, 100 µM of each of the 4 dNTP's, 50 pmol of each primer and 1.25 U Ampli-Taq polymerase (Cetus/Permin Elmer). The final volume was 50 or 100 µl. The incubation mixture was overlaid with a drop of mineral oil to inhibit evaporation.

Two primer combinations were tested: (a) primers SEQ ID NO's:9 and 10, (b) primers SEQ ID NO's:11 and 12.

35 The PCR conditions were also varied. There were tested 1 min 94°C, 1 min 50°C, 1 sec 72°C (30 cycles), and 1 min 94°C,

1 min 60°C, 1 sec 72°C (30 cycles). The PCR was used on isolated DNA and directly on 5 Staphylococcus aureus colonies of an agar plate. Both primer combinations gave the expected product under all the conditions tested.

- 5        The PCR on 5 colonies directly from the agar plate consisting of 30 cycles 1 min 94°C, 1 min 60°C, 1 sec 72°C was also carried out in combination with primers for the protein A gene. These primers consisted of SEQ ID NO's:18 and 19.

- 10        The products formed were analyzed by means of agarose gel electrophoresis. By boiling, 1% (w/s) agarose was dissolved in 50 mM Tris, 50 mM boric acid, 1 mM EDTA (pH 8.3). The gel was poured into a commercially available gel receptacle designed for the purpose. To 10 µl PCR incubation mixture were added 1 µl 40% sucrose, 0.1% bromphenol blue. After the bromphenol  
15        blue had entered the gel over a few cm under the influence of an electric field applied, the result was analyzed under UV light. Since DNA fragments of a known length were also applied to the gel, it could be established whether there had been formed a product of the correct length.

LIST OF REFERENCES

1. Locksley, R.M., Cohen, M.L., Quinn, T.C., Tompkins, L.S., Coyle, M.B., Kirihara, J.M., Counts, G.W.: Annals of Internal Medicine 97, 1982, 317-324.
2. Bradley, J.M., Noone, P., Townsend, D.E., Grubb, W.B.: Lancet 1, 1985, 1493-1495.
3. Sjöström, J.-E., Löfdahl, S., Philipson, L.: Journal of Bacteriology 123, 1975, 905-915.
4. Kornblum, J., Hartmann, B.J., Novick, R.P., Tomasz, A.: European Journal of Clinical Microbiology 5, 1986, 714-718.
5. Beck, W.D., Berger-Bächi, B., Kayser, F.M.: Journal of Bacteriology 165, 1986, 373-378.
6. Matthews, P.R., Reed, K.C., Stewart, P.R.: Journal of General Microbiology 133, 1987, 1919-1929.
7. Matthews, P., Tomasz, A.: Antimicrobial Agents and Chemotherapy 34, Sept. 1990, 1777-1779.
8. Pierre, J., Williamson, R., Borner, M., Gutmann, L.: Antimicrobial Agents and Chemotherapy 34, Sept. 1990, 1691-1694.
9. Stratton, C.W., Gelfand, M.S., Gerberding, J.L.: Antimicrobial Agents and Chemotherapy 34, Sept. 1990, 1780-1782.
10. Tesch, W., Ryffel, C., Strässle, A., Kayser, F.H., Berger-Bächi, B.: Antimicrobial Agents and Chemotherapy 34, Sept. 1990, 1703-1706.
11. Froggatt, J.W., Johnston, J.L., Galetto, D.W., Archer, G.L.: Antimicrobial Agents and Chemotherapy 33, April 1989, 460-466.
12. Archer, G.L., Pennell, E.: Antimicrobial Agents and Chemotherapy 34, Sept. 1990, 1720-1724.
13. Laddaga, R.A., Chu, L., Misra, T.K., Silver, S.: Proceedings National Academy of Sciences USA 84, 1987, 5106-5110.

14. Nucifora, G., Chu, L., Misra, T.K., Silver, S.:  
Proceedings National Academy of Sciences USA 86, 1989,  
3544-3548.
15. Rouch, D.A., Messerotti, L.J., Loo, L.S.L., Jackson, C.A.,  
Skurray, R.A.: Molecular Microbiology 3, 1989, 161-175..
16. Lindberg, M., Sjöström, J.-E., Johansson, T.: Journal of  
Bacteriology 109, 1972, 844-847.
17. Maniatis, T., Fritsch, E.F., Sambrook, J.: Molecular  
cloning; a laboratory manual. Cold Spring Harbor  
Laboratory, Cold Spring Harbor, N.Y., 1982, p. 387.
18. Fluit, A.C., Baas, P.D., Van Boom, J.H., Veeneman, G.H.,  
Jansz, H.S.: Nucleic Acids Research 12, 1984, 6443-6454.
19. Welcher, A.A., Torres, A.R., Ward, D.C.: Nucleic Acids  
Research 1986, 10020-10036.

SEQUENCE LISTSEQ ID NO:1

Nucleotide sequence of the probe from S. aureus strain 05723.

length: 333 nucleotides

GATCCTTATA TACTATCGTG CGTTTCCATG GCCCGCTATT GTACCAAATT AATCGACTCA  
TTGTTGCTTC CTGGGGATAT CCATACTTAC TAATTATGGT TTTTGCCCCT TCCAACGGTG  
GTCCCTGCCA ATAGGACAAA ATGTGATTAA AATGACTATC CATTGCCATA CCTCCTAAAC  
ATTTGCCTAA AAGCATTGTA TGCCCAATAA ATGAATTTTA GGGGTTCTGT TGCAAAGTAA  
AAAAATATAG CTAACCACTA ATTTATCATG TCAGTGTTTCG CTTAACTTGC TAGCATGATG  
CTAATTTCGT GGCATGGCGA AAATCCGTAG ATC

SEQ ID NO:2

DNA sequence for the probe range of S. aureus strain 00646.

length: 333 nucleotides

GATCCTTATA TACTATCGTG CGTTTCCATG GCCCGCTATT GTACCAAATT AATCGACTCA  
TTGTTGCTTC CTGGGGATAT CCATACTTAC TAATTATGGT TTTTGCCCCT TCCAACGGTG  
GTCCCTGCCA ATAGGACAAA ATGTGATTAA AATGACTATC CATTGCCATA CCTCCTAAAC  
ATTTGCCTAA AAGCATTGTA TGCCCAATAA ATGAATTTTA GGGGTTTGTG TGCAAAGTAA  
AAAAATATAG CTAACCACTA ATTTATCATG TCAGTGTTTCG CTTAACTTGC TAGCATGATG  
CTAATTTCGT GGCATGGCGA AAATCCGTAG ATC

SEQ ID NO:3

DNA sequence for the probe range of S. aureus strain A216.

length: 332 nucleotides

GATCCTTATA TACTATCGTG CGTTTCCATG GCCCGCTATT GTACCAAATT AATCGACTCA  
TTGTTGCTTC CTGGGGATAT CCATACTTAC TAATTATGGT TTTTGCCCCT TCCAACGGTG  
GTCCCTGCCA ATAGGACAAA ATGTGATTAA AATGACTATC CATTGCCATA CCTCTTAAAC  
ATTTGCCTAA AAGCATTGTA TGCCCAATAA ATGAATTTTA GGGGTTCTGT TGCAAAGTAA  
AAAATATAGC TAACCACTAA TTTATCATGT CAGTGTTTCG TTAACCTTGGT AGCATGATGC  
TAATTTTCGT GCATGGCGAA AATCCGTAGA TC

SEQ ID NO:4

DNA sequence for the probe range of S. aureus strain 02599.

length: 264 nucleotides

GATCTTATAT ACTATCGTGC GTTTCCATGG CCCGCTATTG TACCAAATTA ATCGTCTCAT  
TGTTGCTTCC TGGGGATATC CATACTTACT AATTATGGTT TTTGCCCCTT CCAACGGTGG  
TCCCTGCCAA TAGGACAAAA TGTGATTTAA ATGGGTTCTG TTGCAAAGTA AAAAAATATA  
GCTAACCACCT AATTTATCAT GTCAGTGTTG GCTTAACTTG CTAGCATGAT GCTAATTTTCG  
TGGCATGGCG AAAATCCGTA GATC

SEO ID NO:5DNA sequence for the probe range of S. aureus strain 214.

length: 263 nucleotides

GATCCTTATA TACTATCGTG CGTTTCCATG GCCCGCTATT GTACCAAATT AATCGTCTCA  
TTGTTGCTTC CTGGGGATAT CCATACTTAC TAATTATGGT TTTTGCCCCT TCCAACGGTG  
GTCCCTGCCA ATAGGACAAA ATGTGATTTA AATGGGTTCT GTTGCAAAGT AAAAAAATAT  
AGCTAACCAC TAATTTATCA TGTCAGTGTT CGCTTAACTT GCTAGCATGA TGCTAATTTT  
GTGGCATGGC GAAAATCCGG ATC

SEO ID NO:6DNA sequence for the probe range of S. aureus strain 215C.

length: 264 nucleotides

GATCCTTATA TACTATCGTG CGTTTCCATG GCCCGCTATT GTACCAAATT AATCGTCTCA  
TTGTTGCTTC CTGGGGATAT CCATACTTAC TAATTATGGT TTTTGCCCCT TCCAACGGTG  
GTCCCTGCCA ATAGGACAAA ATGTGATTTA AATGGGTTCT GTTGCAAAGT AAAAAAATAT  
AGCTAACCAC TAATTTATCA TGTCAGTGTT CGCTTAACTT GCTAGCATGA GCTAATTTTC  
TGGCATGGCG AAAATCCGTA GATC

SEO ID NO:7DNA sequence for the probe range of S. aureus strain 06231.

length: 265 nucleotides

GATCCTTATA TACTATCGTG CGTTTCCATG GCCCGCTATT GTACCAAATT AATCGTCTCA  
TTGTTGCTTC CTGGGGATAT CCATACTTAC TAATTATGGT TTTTGCCCCT TCCAACGGTG  
GTCCCTGCCA ATAGGACAAA ATGTGATTTA AATGGGTTCT GTTGCAAAGT AAAAAAATAT  
AGCTAACCAC TAATTTATCA TGTCAGTGTT CGCTTAACTT GCTAGCATGA TGCTAATTTT  
GTGGCATGGC GAAAATCCGT AGATC

SEO ID NO:8DNA sequence for the probe range of S. aureus strain 1335.

length: 265 nucleotides

GATCCTTATA TACTATCGTG CGTTTCCATG GCCCGCTATT GTACCAAATT AATCGTCTCA  
TTGTTGCTTC CTGGGGATAT CCATACTTAC TAATTATGGT TTTTGCCCCT TCCAACGGTG  
GTCCCTGCCA ATAGGACAAA ATGTGATTTA AATGGGTTCT GTTGCAAAGT AAAAAAATAT  
AGCTAACCAC TAATTTATCA TGTCAGTGTT CGCTTAACTT GCTAGCATGA TGCTAATTTT  
GTGGCATGGC GAAAATCCGT AGATC

SEO ID NO:9

DNA sequence of PCR primer.

length: 20 nucleotides

TTAATCGACT CATTGTTGCT

SEO ID NO:10

DNA sequence of PCR primer.

length: 20 nucleotides

TTTCGCCATG CCACGAAATT

SEO ID NO:11

DNA sequence of PCR primer.

length: 20 nucleotides

GATCCTTATA TACTATCGTG

SEO ID NO:12

DNA sequence of PCR primer.

length: 20 nucleotides

GATCTACGGA TTTTCGCCAT

SEO ID NO:13

DNA sequence of PCR primer.

length: 20 nucleotides

CGTGGCATGG CGAAAATCCG

SEO ID NO:14

DNA sequence of PCR primer.

length: 20 nucleotides

TTACTTTGCA ACAGAACCCC

SEO ID NO:15

DNA sequence of probe.

length: 20 nucleotides

TAACTTGCTA GCATGATGCT

SEO ID NO:16

DNA sequence of probe.

length: 20 nucleotides

TTTATCATGT CAGTTGTTTCGC

SEO ID NO:17

DNA sequence of protein A-specific PCR primer  
length: 20 nucleotides

GCGCAACACG ATGAAGCTCA

SEO ID NO:18

DNA sequence of protein A-specific PCR primer  
length: 20 nucleotides

CTTTTGGTGC TTGAGCATCG

SEO ID NO:19

DNA sequence of protein A-specific PCR primer  
length: 20 nucleotides

CTGCGCAACA CGATGAAGCT

## CLAIMS

1. A DNA fragment derived from a methicillin-resistant Staphylococcus strain and consisting essentially of or being complementary to the nucleotide sequence shown in the sequence list under SEQ ID NO:1 or a related nucleotide sequence, such  
5 as one of the nucleotide sequences shown in the sequence list under SEQ ID NO's:2-8, or part of such a nucleotide sequence having a length of at least 14 nucleotides.
2. A DNA probe for the detection of Staphylococcus bacteria that are resistant to methicillin and related  
10 penicillins insensitive to  $\beta$ -lactamase, comprising a DNA fragment according to claim 1.
3. A DNA primer for DNA amplification by means of a polymerase chain reaction, comprising a DNA fragment according to claim 1.
- 15 4. A plasmid consisting essentially of a bacterial cloning vector including an insertion consisting of a DNA fragment according to claim 1.
5. Bacteria containing at least one plasmid according to claim 4.
- 20 6. A diagnostic test kit for the detection in a sample of Staphylococcus bacteria that are resistant to methicillin and related penicillins insensitive to  $\beta$ -lactamase by means of hybridization analysis, comprising at least a DNA probe according to claim 2 as well as one or more further means  
25 required for hybridization analysis, such as a denaturation liquid, a hybridization liquid, a washing liquid, a solid carrier, a hybridization vessel and label detecting means.
7. A process for the detection in a sample of Staphylococcus bacteria that are resistant to methicillin and  
30 related penicillins insensitive to  $\beta$ -lactamase by means of hybridization analysis, which comprises isolating the DNA from the bacteria in the sample, contacting the isolated DNA under hybridization conditions with at least a DNA probe according

to claim 2, washing nonhybridized probe and analyzing any hybridization by label detection.

8. A diagnostic test kit for the detection in a sample of Staphylococcus bacteria that are resistant to methicillin and related penicillins insensitive to  $\beta$ -lactamase by means of PCR analysis, comprising a set of DNA primers according to claim 3, as well as one or more further means required for PCR analysis, such as a polymerase, a polymerization liquid, an oil overlay, a reaction vessel and means for detecting the amplified DNA.


9. A diagnostic test kit according to claim 8, which, in addition to a set of primers according to claim 3, also contains a set of protein A-specific primers.

10. A process for the detection in a sample of Staphylococcus bacteria that are resistant to methicillin and related penicillins insensitive to  $\beta$ -lactamase by means of PCR analysis, which comprises isolating, if required, the DNA from the bacteria in the sample, subjecting the DNA that may or may not have been isolated to several PCR cycles using a set of DNA primers according to claim 3, and analyzing the amplified DNA.

11. A process according to claim 10, also comprising the use in the PCR cycles of a set of protein A-specific primers, in addition to a set of primers according to claim 3.

# INTERNATIONAL SEARCH REPORT

International Application No PCT/NL 90/00178

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (If several classification symbols apply, indicate all) *		
According to International Patent Classification (IPC) or to both National Classification and IPC		
IPC <sup>5</sup> : C 12 Q 1/68, C 12 N 15/31, C 12 N 15/10		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched ?		
Classification System	Classification Symbols	
IPC <sup>5</sup>	C 12 Q, C 12 N	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched *		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT</b> *		
Category *	Citation of Document, ** with indication, where appropriate, of the relevant passages **	Relevant to Claim No. **
X	Antimicrobial Agents and Chemotherapy, vol. 33, no. 4, April 1989, American Society for Microbiology, J.W. Froggatt et al.: "Antimicrobial resistance in nosocomial isolates of Staphylococcus haemolyticus", pages 460-466 see page 461, right-hand column, lines 22-54 (cited in the application)	1, 2, 4-9
X	Proc. Natl. Acad. Sci. USA, vol. 84, August 1987, R.A. Laddaga et al.: "Nucleotide sequence and expression of the mercurial-resistance operon from Staphylococcus aureus plasmid pI258", pages 5106-5110 see figure 2, nucleotides 6071-6403 (cited in the application)	1, 3, 4
<p>* Special categories of cited documents: **</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"Z" document member of the same patent family</p>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
18th March 1991	24. 04. 91	
International Searching Authority	Signature of Authorized Officer	
EUROPEAN PATENT OFFICE	F.W. HECK 	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, ** with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	<p>Journal of General Microbiology, vol. 133, 1987, SGM, P.R. Matthews et al.: "The cloning of chromosomal DNA associated with methicillin and other resistances in staphylococcus aureus", pages 1919-1929 see page 1923, last paragraph; page 1924 (cited in the application)</p> <p>-----</p>	1,2,4-9